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CONCISE REVIEW

Reemergence of neural crest stem cell-like states in melanoma during disease progression and treatment

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Abstract

Melanoma is the deadliest of all skin cancers due to its high metastatic potential. In recent years, advances in targeted therapy and immunotherapy have contributed to a remarkable progress in the treatment of metastatic disease. However, intrinsic or acquired resistance to such therapies remains a major obstacle in melanoma treatment. Melanoma disease progression, beginning from tumor initiation and growth to acquisition of invasive phenotypes and metastatic spread and acquisition of treatment resistance, has been associated with cellular dedifferentiation and the hijacking of gene regulatory networks reminiscent of the neural crest (NC)—the developmental structure which gives rise to melanocytes and hence melanoma. This review summarizes the experimental evidence for the involvement of NC stem cell (NCSC)-like cell states during melanoma progression and addresses novel approaches to combat the emergence of stemness characteristics that have shown to be linked with aggressive disease outcome and drug resistance.

KEYWORDS

neural crest stem cells (NCSCs), development, cancer, melanoma, tumor initiation, invasion, drug resistance

1 | INTRODUCTION

The neural crest (NC) is a transient and multipotent stem cell population arising at the dorsal neural tube at early stages of vertebrate development.^{1–3} Neural crest stem cells (NCSCs) disseminate into the embryo to differentiate into an array of cell lineages with remarkably different functions.⁴ Among the adult NC derivatives are cartilage and bone structures in the head, the outflow tract of the heart, enteric nervous system cells in the gut, neuronal and non-neuronal cells of the peripheral nervous system, melanocytes and others.^{5,6}

While most of the NC progeny have acquired a fully differentiated fate in the adult, cells with NCSC-like characteristics have been isolated from several adult mouse tissues⁷ as well as human skin^{8–12} and dental pulp.¹³ Even though the isolated cells possessed multipotent capacities in vitro and could give rise

to different NC lineages, the question remains whether NCSC-like cells derived from adult tissues really represent a maintained embryonic stem cell niche or whether these cells reacquire stem cell features due to isolation procedures and cultivation in vitro.

The reemergence of NCSC-like cell states in vivo has been observed and studied in the context of pathological and physiological conditions such as carcinogenic transformations of NC-derived adult tissues or wound healing, respectively. Murine tissue injury models revealed that NC-derived Schwann cells (peripheral glia) dedifferentiate after injury and reacquire a progenitor-like cell state by downregulating genes crucial for the myelination machinery and upregulating NCSC-associated factors such as the neurotrophin receptor CD271 (also p75^{NTR}, NGFR).^{14,18} Injury-activated glial cells were further shown to crucially assist regeneration of full-thickness skin wounds or amputated digit tips.^{17,18}

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Furthermore, previous reports revealed that Schwann cell precursors (SCPs) during embryonic development harbor NC cell features and are able to generate a variety of neural and non-neural cell types.^{15,16} Likewise, carcinogenic lesions of adult NC-derived tissues such as melanoma or neuroblastoma can both present with cells expressing NCSC-associated factors that are not normally expressed in the healthy adult tissue.¹⁹⁻²¹ This review focuses on melanoma, wherein the aberrant regulation or expression of developmental NCSC genes or pathways is associated with different aspects of malignancy (Figure 1), such as tumor initiation and sustained tumor growth,²²⁻²⁵ promotion of metastatic spread,²⁶ as well as resistance to therapies²⁷⁻³⁰ and immune evasion.^{31,32}

Significance statement

This review summarizes the experimental evidence for reemergence of neural crest stem cell (NCSC)-like cells in melanoma, which hijack embryonic programs to acquire advantages regarding tumor initiation and growth, metastatic spread, and eventually therapy resistance. Furthermore, the authors discuss preclinical efforts to specifically target NCSC-like melanoma cells to combat drug resistance, which is a major goal in the field and will hopefully soon improve melanoma therapy for patients.

2 | DEVELOPMENTAL NCSC GENES REGULATE MELANOMA GROWTH AND HOMEOSTASIS

Cutaneous malignant melanoma, the most aggressive skin cancer,³³ can present with astonishing heterogeneity, ranging from cells expressing typical melanocyte differentiation genes to gene sets typical for other NC-derived cell lineages like neurons or glia, among others.³⁴⁻³⁶ The origin of such heterogeneous tumors has been

heavily debated and research was carried out on the expression of factors reminiscent of stem cells, which equips melanoma cells with renewal capacities in vitro and tumor formation potential in vivo.^{22,37,38} For instance, the neurotrophin receptor CD271, which has been used for isolation of rat NCSCs with multipotent capacities in vitro,¹⁴ was found reexpressed in a subset of melanoma cells that were able to initiate tumor formation when grafted into immunocompromised mice, while CD271-negative cells lacked such potential²²

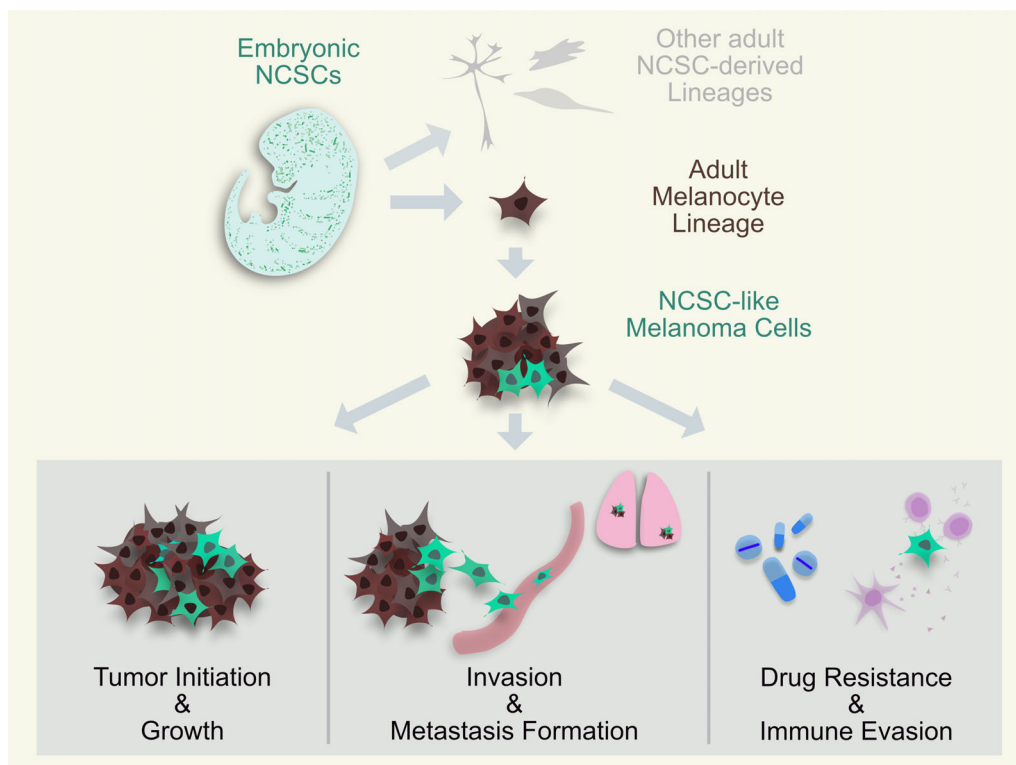


FIGURE 1 Reacquisition of neural crest stem cell (NCSC)-like characteristics in melanoma and its implications. Embryonic NCSCs (dark green) arise at the developing dorsal neural tube and migrate into the whole embryo to form different tissues such as craniofacial bone and cartilage, enteric and peripheral nervous system cells (light grey) and, amongst many others, also cells of the melanocyte lineage, specifically melanocyte stem cells (MeSCs), melanoblasts and melanocytes (brown). Due to malignant transformations, those cells can progress into melanoma. Different studies have shown that some melanoma cells (light green) can hijack embryonic NCSC programs, bestowing them with different advantageous characteristics. Melanoma cells with reacquired NCSC features have been associated with the ability to form novel tumors and sustain growth, increased cell invasiveness and metastasis formation, as well as the ability to resist different melanoma therapies and evade immune surveillance

(Table 1). Furthermore, the tumors grown from single grafted CD271^{high} cells presented with the heterogeneity of the parental tumor from which they were isolated, and not exclusively of CD271-positive cells as grafted to generate the tumor, supporting the idea that CD271^{high} cells represent stem cell-like cells.²² Of importance, CD271 is expressed in few adult cell types^{64,65} and exerts divergent functions depending on the tissue context, for instance in melanocytes versus transformed malignant melanoma.^{66,67} While signal transduction networks related to melanocytic differentiation (such as skin development, metabolic hormone processes or cell adhesion) have been found enriched in CD271-positive human melanocytes, the same pathways were significantly downregulated in CD271-positive human melanoma cells,⁶⁶ supporting the association of CD271^{high} cell states with dedifferentiation and stemness potential in melanoma.

The idea that stem cell-like cells exist in melanoma was further strengthened by the finding that apart from CD271, the expression of other genes reminiscent of NCSCs was reported in melanoma and found to be essential for tumor initiation and maintained tumor growth. The transcription factor (TF) SRY-related HMG-box 10 (SOX10), for instance, which regulates the melanocytic and glial murine lineages derived from the NC,^{39,40} was found highly expressed in melanocytic nevi and malignant melanoma.^{24,68,69} Upon depletion of SOX10 in a transgenic *NRAS*^{Q61K}-mutant, *Ink4a*-deficient murine melanoma model, tumor growth was diminished, and silencing of SOX10 in human melanoma cells drastically reduced the number of cells expressing CD271 and completely abolished tumor growth in vivo²⁴ (Table 1). Similar to SOX10, the TF Ying Yang 1 (YY1) was found to regulate both murine NC development and melanoma formation in *NRAS*^{Q61K}-mutant, *Ink4a*-deficient mice²⁵ (Table 1). The importance of reemerging NCSC-like cell states during melanoma initiation was highlighted in yet another study, where in *BRAF*^{V600E}-mutant, *p53*-deficient melanoma-prone zebrafish, cells capable of melanoma initiation activated a gene regulatory program characterized by the activation of zebrafish embryonic NC regulators such as *crestin*²³ (Table 1). Similarly, a recent study identified a set of genes expressed in embryonic melanoblasts, which are downregulated in fully differentiated melanocytes but partially reexpressed in human metastasis samples, providing further evidence for melanoma cells reacquiring progenitor-like cell states.⁷⁰ Consequently, some of those genes like *KDELR3* indeed proved to be essential for the formation of experimental metastasis in a model of tail vein-injected murine B16 cells.⁷⁰

Nevertheless, the role of some genes involved in NC development remains unclear and heavily debated. SOX2, a member of the SRY TF family like SOX10, for instance, was shown to regulate NCSCs and SCPs together with MITF, while SOX2 downregulation led to precursor differentiation into melanocytes,⁷¹ suggesting that the upregulation of SOX2 in melanoma might orchestrate stem cell-like traits. However, while some studies report that SOX2 promotes tumor initiation of human cells grafted into immunocompromised mice,^{72,73} others have shown that it is dispensable for melanomagenesis in genetically engineered mouse models.^{74,75}

While some factors reminiscent of NC development regulate cell cycle regulatory networks to allow tumor initiation and maintenance

of adult melanoma, other cellular processes enabling melanoma tumor formation such as transcriptional regulation^{44,45} or metabolism²⁵ have been shown to be regulated by NCSC-associated factors. It was shown, for instance, that dihydroorotate dehydrogenase (DHODH), an enzyme involved in nucleotide biosynthesis, is essential for transcriptional elongation of genes crucial for both NC development and melanoma formation and that hence, inhibition of DHODH suppressed proper NC development as well as melanoma formation, especially when combined with *BRAF* inhibition⁴⁴ (Table 1). Mechanistically, DHODH inhibition-induced nucleotide stress has recently been linked to the RNA helicase DDX21, which senses nucleotide shortage in NCSCs as well as melanoma cells, upon which it stops transcriptional elongation⁴⁵ (Table 1). In addition, DDX21 was found to regulate transcription in melanocyte stem cells (McSCs) in the hair follicle bulge to prevent differentiation into melanocytes.⁷⁶

There has been much debate recently about whether the cell at the origin of melanoma indeed represents a stem cell-like cell. In fact, the above-mentioned studies somewhat stand in contrast to reports claiming that instead of cells with stem cell features, fully differentiated melanocytes can give rise to melanoma. For instance, it was shown that *BRAF*^{V600E}-mutant, *Pten*-deficient fully differentiated and melanin-producing melanocytes could give rise to melanoma.⁷⁷ Of importance, Köhler et al⁷⁷ state in their study that after a first radial growth phase initiated by fully differentiated melanocytes, a subset of melanoma cells also underwent a transcriptional dedifferentiation process potentially reminiscent of the reemergence of NCSC-like programs reported by others.

Yet another theory on the origin of melanoma argues that McSCs residing in the bulge of the hair follicle, rather than differentiated melanocytes, could stand at the origin of melanoma primary tumor formation.^{78,79} Moreover, given that adult Schwann cells have the capacity of dedifferentiation in vivo¹⁸ and that SCPs are able to generate melanocytes,⁸⁰ it is conceivable that some melanoma might derive from the peripheral glial lineage.

Several animal models have allowed melanoma induction with specific cues followed by in-depth analysis and lineage tracing to understand disease initiation and progression. However, such analyses remain challenging in the context of human disease. While single-cell studies of patient-derived melanoma allow unprecedented insights into heterogeneous disease evolution, an alternative approach to tackle melanoma origin as such, could be the artificial induction of melanoma in melanocyte lineage cells derived from human embryonic stem cells followed by lineage tracing and single-cell characterization. Yet, the mystery of how human melanoma arises in vivo remains to be unraveled.

3 | NCSC-LIKE CELLS CAN INDUCE INVASION AND METASTATIC SPREAD OF MELANOMA

Apart from inducing or maintaining melanoma tumor growth, the reacquisition of NCSC-like characteristics has also been associated

TABLE 1 Literature overview of NCSC-associated factors regulating parts of melanomagenesis

Melanoma implication	Factor (function)	Detailed function in melanoma	References	NCSC function	References
Tumor Initiation/growth	SOX10 (TF)	Broadly expressed in human nevi and melanomas. Depletion leads to abolished tumor formation in a <i>NRAS^{Q61K} Ink4a</i> -deficient mouse model, associated with reduced numbers of CD271 ⁺ cells.	24	Specifies murine NCSCs and melanocytic and glial lineages.	39,40
	YY1 (TF)	Haploinsufficiency is enough to prevent melanoma initiation in a <i>NRAS^{Q61K} Ink4a</i> -deficient mouse model. Regulates shared metabolic and translational pathways in neural crest and melanoma.	25	Essential for early murine NC development and the adult melanocyte lineage.	25
	CD271/NGFR/p75 ^{NTR} (receptor tyrosine kinase)	Single CD271 ⁺ melanoma patient-derived cells can form tumors (with the heterogeneity of the parental tumor) upon grafting into immunocompromised mice, while CD271 ⁻ cells cannot. Inhibition of CD271 in human melanoma cells reduces their tumor initiation potential.	22,41	Used to isolate mammalian NCSCs that were multipotent in vitro	14
	EZH2 (histone methyl transferase)	Upregulated in human malignant melanoma compared to melanocytes. Depletion in a <i>NRAS^{Q61K} Ink4a</i> -deficient mouse model inhibits melanoma growth.	42	Controls differentiation of NC-derived mesenchymal lineages (bone and cartilage)	43
	DHODH (pyrimidine metabolism)	Transcriptional elongation of genes crucial for melanomagenesis.	44	Transcriptional elongation of neural crest developmental genes.	44
	DDX21 (RNA helicase)	Controls transcriptional elongation (after nucleotide shortage-induced stress).	45	Controls transcriptional elongation.	45
Phenotype switch/invasion	Crestin (unknown)	Marks tumor-initiating cells in a <i>BRAF^{V600E} p53</i> -deficient zebrafish melanoma model.	23	mRNA widely expressed in zebrafish NCSCs	46
	MSX1 (TF)	Induces phenotypic switching (E-cadherin ^{high} , nonmigratory toward ZEB1 ^{high} , invasive) in melanoma. Depletion reduces liver metastasis after tail vein injection of human melanoma cells into immunocompromised mice.	47	NC induction in xenopus	48
	Twist1/Zeb1 (TF)	<i>NRAS/BRAF</i> activation in melanocytes leads to upregulation of EMT TFs including TWIST1 and ZEB1, dedifferentiation and neoplastic transformation of melanocytes.	49	Twist1: NC specifier; delamination of cranial NC; cell fate decision within cardiac NC Zeb1: upregulated by Zeb2, essential for melanocyte migration and differentiation.	50-52
	FOXD3 PAX3 (TF)	FOXD3 and PAX3 drive CXCR4 expression in melanoma, which was shown to promote melanoma metastasis formation.	53,54,55	FOXD3: NC specifier PAX3: expressed in neural plate border	56,57
	CD271 (receptor tyrosine kinase)	Associated with increased metastasis in patients. Transient	35,26		

TABLE 1 (Continued)

Melanoma implication	Factor (function)	Detailed function in melanoma	References	NCSC function	References
		overexpression induces a reversible phenotype switch in vitro and increased metastatic potential of human melanoma cells grafted onto immunocompromised mice.			
	EZH2 (histone methyl transferase)	Depletion in a <i>NRAS</i> ^{Q61K} <i>Ink4a</i> -deficient mouse model leads to abolished macro-metastasis formation.	42		
Drug Resistance	FOXD3 (TF) ERBB3 (receptor tyrosine kinase)	FOXD3 upregulates ERBB3, leading to BRAFi resistance in vitro and in vivo.	58	ERBB3: NC differentiation and dev. of sympathetic nervous system	59
	CD271 (receptor tyrosine kinase)	NGFR ⁺ AXL ⁺ melanoma patient cells represent a dormant, MAPKi-resistant cell population. Long-term (3 weeks) BRAFi treatment leads to emergence of a drug-tolerant or drug-resistant NC-like cell state in vitro.	60,61		
	RXRG (nuclear receptor)	Minimal residual disease in a BRAFi/MEKi-tolerant PDX model represents as dedifferentiated melanoma (NGFR ⁺ RXRG ⁺ AQP1 ⁺ GFRA ⁺).	28	Expressed in migrating cranial chick NC cells.	62
Immune Evasion	CD271 (receptor tyrosine kinase)	TNF α induces dedifferentiation of melanoma cells (NGFR ^{high}) and resistance to adaptive T-cell therapy in a murine model of adoptive cell transfer therapy. Long-term exposure of patient-derived melanoma cells to antigen-specific cytotoxic T cells leads to enrichment of NGFR ^{high} cells, which are refractory to T cells as well as to BRAF/MEKi	32,31		
	EZH2 (histone methyl transferase)	Intratumoral TNF α and T-cell accumulation induce Ezh2 in melanoma cells originating from <i>NRAS</i> ^{Q61K} <i>Ink4a</i> -loss or B16 F10 murine models, leading to loss of immunogenicity.	63		

Abbreviations: MEK, mitogen-activated extracellular signal-regulated kinase; NC, neural crest; NCSC, neural crest stem cell; SOX10, SRY-related HMG-box 10; TF, transcription factor; YY1, Ying Yang 1. Transgenic animal models: *NRAS*^{Q61K} *Ink4a*-deficient mouse model, *Tyr:Nras*^{Q61K} *Cdkn2a*^{-/-}; *BRAF*^{V600E} *p53*-deficient zebrafish, *mitfa*:*BRAF*^{V600E} *p53*^{-/-}.

with later stages of melanoma progression, such as invasiveness and metastatic spread.^{22,26,35} To become invasive and disseminate from the primary tumor, cancer cells are thought to undergo an epithelial to mesenchymal transition (EMT), rendering them with decreased cell-to-cell contacts, increased motility, and an increased potential to remodel extracellular matrix components.^{81,82}

Although, by definition, classical EMT is a process associated with epithelial cancers, which is not the case for melanoma, melanoma cells can undergo an EMT-like process called “phenotype

switching,” where cells transform from a high proliferative/low invasive to a low proliferative/high invasive phenotype.^{83,84} Even though it has been reported that melanoma invasion and metastasis can progress independently of the ‘classical’ phenotype switching model,⁸⁵ EMT-like phenotype switching is thought to be a crucial driver of melanoma invasiveness and metastasis formation.⁸⁶

Migratory NCSCs delaminating from the neural tube to migrate out into the embryo are a paradigm example for EMT during

embryonic development.^{4,5,87} Intriguingly, regulatory genes responsible for developmental NC EMT are reexpressed in adult malignant melanoma,^{19,88} including members of the Snail, Zeb, and Twist families⁴⁹ (Table 1). Caramel et al⁴⁹ showed that upon *NRAS/BRAF* activation in melanocytes, the EMT TFs *TWIST1* and *ZEB1* were upregulated and induced dedifferentiation and neoplastic transformation. They also showed that this EMT-TF signature, when found in late-stage melanoma patients, correlated with poor prognosis. Similarly, transforming growth factor beta also acts as a potent inducer of melanoma phenotype switching,^{84,89} while playing a crucial physiological role in NC development by providing signaling cues for migration and differentiation into several lineages.⁹⁰⁻⁹²

Apart from an invasive and migratory potential, epithelial cancer cells undergoing EMT have been associated with the acquisition of stem cell-like features.^{93,94} Similarly, melanoma phenotype switching has been associated with the reemergence of signatures similar to NCSC regulatory networks⁴⁷ (Table 1). Expression of the NCSC-associated factors *CD271* and *SOX10*^{39,40} in human melanoma correlates with high metastatic potential and worse patient prognosis³⁵ (Table 1). Furthermore, *Msh* homeobox 1 (*MSX1*), which specifies the NC at the neural border of zebrafish and xenopus,⁴⁸ when upregulated in melanoma, leads to dedifferentiation of melanoma cells, which upregulate NCSC-associated factors such as *CD271* and induce a phenotype switch toward increased cell migration⁴⁷ (Table 1). Vice versa, silencing of *MSX1* reduces liver metastasis of tail vein-injected human melanoma cells in mice.⁴⁷ Also the zebrafish and murine NC specifier *FOXD3* together with *PAX3*, which is expressed at the neural plate border,^{56,57} have been shown to induce human melanoma invasiveness by directly regulating *CXCR4*,⁵³ which in turn regulates melanoma metastasis formation^{54,55} (Table 1). Finally, even though a zebrafish study suggested otherwise,⁹⁵ ectopic overexpression of *CD271* induces a phenotype switch in human melanoma cells, ultimately leading to an increased metastatic potential of human melanoma cells grafted into immunocompromised mice²⁶ (Table 1). These findings revealed a functional involvement of single factors reminiscent of NCSCs in melanoma disease progression. Whether, in general, the reemergence of a broader NCSC signature is functionally implicated in melanoma metastasis formation remains to be elucidated.

4 | DEDIFFERENTIATED MELANOMA CELLS DISPLAY RESISTANCE TO DIFFERENT THERAPIES

While traditionally the most common therapy for melanoma has been surgical removal of primary tumors plus radiation and chemotherapy,⁹⁶ the advent of immune and targeted therapies significantly improved the survival rate, especially of patients with metastatic melanoma.³³ Targeted therapies for melanoma are mostly directed against the serine/threonine kinase *BRAF* or the mitogen-activated extracellular signal-regulated kinase (*MEK*), leading to inhibition of the mitogen-activated protein kinase (*MAPK*) pathway, an

oncogenic pathway mutated and constitutively active in most melanomas.^{97,98} Immunotherapies on the other hand aim at boosting the antitumoral activity of cytotoxic T lymphocytes (CTLs) to combat melanoma.²⁹ However, one of the major remaining challenges is the acquisition or preexistence of melanoma cells resistant to such therapies,⁹⁹⁻¹⁰³ which ultimately lead to relapse.

Resistance to different melanoma therapies has been associated with cells undergoing phenotype switching and lacking pigmentation-related differentiation genes while expressing genes reminiscent of NC development.^{28-30,32,104,105} Specifically, *MAPK* pathway inhibition was shown to promote the de novo generation or expansion of subpopulations of melanoma cells expressing NCSC-associated factors like *CD271*^{28,106} or the NC specifier gene *FOXD3*.⁵⁸ In addition, targeted therapy led to increased expression of genes linked to invasiveness like the receptor tyrosine kinase *AXL*^{106,107} (Table 1). NCSC-like melanoma cell subpopulations were further reported to contribute to minimal residual disease and, ultimately, to disease relapse.^{28,108} Conversely, another study showed that melanoma cells expressing the melanocyte differentiation gene *Dopachrome tautomerase* (*DCT*, also *TYRP2*) were intrinsically resistant to *BRAF* inhibition.¹⁰⁹ However, increasing evidence supports the hypothesis that it is particularly the dedifferentiated melanoma cell population, expressing genes reminiscent of NCSCs, that can resist different treatments, including immunotherapies.

Along that line, a recent study showed that long-term exposure of patient-derived melanoma cells to antigen-specific (*MART1*) T cells led to an enrichment of *CD271*^{high} melanoma cells, which showed increased resistance to cytotoxic T cells (which recognized differentiation and non-differentiation antigens), as well as to *BRAF* and *MEK* inhibitors³¹ (Table 1). In line with these findings, another study revealed that in a mouse adoptive T cell therapy model, tumor necrosis factor alpha (*TNFα*)-induced inflammation led to dedifferentiation of patient-derived transplanted melanoma by upregulation of *CD271* and downregulation of melanocyte-specific antigens, which resulted in reduced tumor recognition by infiltrating T cells³² (Table 1). Furthermore, while Boshuizen et al³¹ interfered with *CD271* upregulation to combat T cell resistance and relapse in their preclinical model via an unspecific heat shock protein inhibitor, another study developed a *CD271*-specific monoclonal humanized antibody to counteract *CD271* function.¹¹⁰ The authors showed that treatment of *CD271*-positive human melanoma grafts in NOD/scid mice with this *CD271* antibody in combination with natural killer or peripheral blood mononuclear effector cells achieved a significant antitumor effect.¹¹⁰ Whether such a *CD271*-specific antibody could combat therapy resistance of melanoma tumors toward targeted or immune therapy remains to be answered, but previous findings appear to support such an approach.^{28,31,32}

Immunotherapy-induced T-cell accumulation and *TNFα* have also been shown to induce other factors reminiscent of NC development in melanoma, such as the histone methyltransferase *Ezh2*, which regulates mesenchymal fates during murine NC development.⁴³ *Ezh2* upregulation in melanoma led to reduced immunogenicity of B16 F10

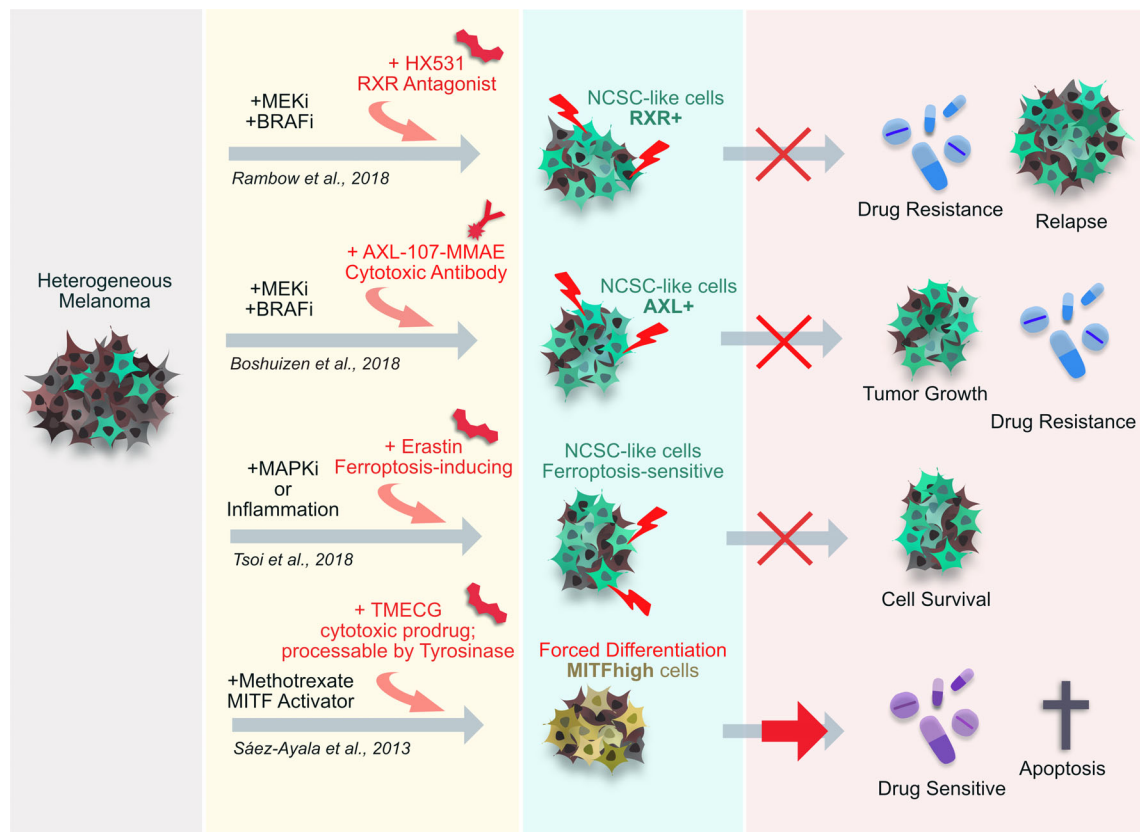


FIGURE 2 Interfering with dedifferentiated, neural crest stem cell (NCSC)-like melanoma cells to combat drug resistance. Different preclinical studies successfully managed to circumvent therapy resistance of melanoma cells or patient-derived grafts in mice by targeting the reemergence of NCSC-like melanoma states. Rambow et al²⁸ were able to attenuate the accumulation of NCSC-like cells after BRAF/MEK inhibition by treating patient-derived melanoma with an antagonist toward RXRG, a NCSC-associated gene strongly upregulated within their drug resistant melanoma subpopulation. Similarly, Boshuizen et al¹⁰⁷ successfully targeted tumor growth by combining MAPK inhibition with a cytotoxic antibody against AXL^{high} cells emerging as resistant cells after BRAF/MEK inhibition. AXL is associated with invasiveness and drug resistance in melanoma⁶⁰ but has also been associated with reemergence of NCSC states in melanoma.¹⁰⁶ Also, Tsoi et al¹⁰⁶ could show that MAPK inhibition as well as pro-inflammatory signaling from immunotherapies promoted drug resistant, dedifferentiated melanoma cells, which were characterized by increased sensitivity to ferroptosis. The authors subsequently co-treated melanoma cells with targeted therapy and the ferroptosis-inducing drug Erastin, which led to decreased melanoma cell survival. Sáez-Ayala et al¹¹¹ achieved to circumvent drug resistance by forced differentiation of melanoma cells due to treatment with methotrexate (MTX), which induced the expression of the melanocyte differentiation marker MITF and inhibited invasiveness. This drug was further combined with the cytotoxic prodrug TMECG, activated by tyrosinase (a target of MITF), which is expressed in differentiated melanocytes

or *Nras*^{Q61K}-mutant *Ink4a*-deficient murine tumors autologously grafted onto C57BL/6 mice, while pharmacological inhibition of Ezh2 attenuated this effect and synergized with anti-CTLA-4 and IL-2 immunotherapies in mice⁶³ (Table 1).

5 | TARGETING THE REEMERGENCE OF NCSC-LIKE MELANOMA STATES

In line with NCSC-like cell states observed in melanoma during disease progression and treatment, increasing evidence supports approaches targeting melanoma cells that have hijacked developmental programs. Indeed, several preclinical studies have succeeded in targeting or inhibiting the emergence of dedifferentiated melanoma cells to combat therapy resistance (Figure 2).

For instance, inhibiting CD271 in melanoma cells restored their susceptibility to BRAF inhibitors.²⁷ Likewise, suppressing the emergence of dedifferentiated, NCSC-like melanoma cells upon MEK and BRAF inhibition (MEKi and BRAFi) interfered with resistance formation *in vivo*.²⁸ Specifically, this elegant study showed that a set of NCSCs genes, such as the Retinoid X receptor gamma (RXRG), which is expressed in chicken NCSCs,⁶² was induced by MEKi and BRAFi treatment, and that pharmacological inhibition of RXRG prevented disease relapse of patient-derived melanoma in immunocompromised mice²⁸ (Figure 2). Yet another study used a cytotoxic antibody approach to target AXL^{high} melanoma cells resistant to MEKi and BRAFi, which led to reduced tumor growth and restored drug susceptibility¹⁰⁷ (Figure 2). Of note, AXL has not been identified as a regulator of the embryonic NC as such, however its expression has been associated with NCSC-reminiscent factors such as CD271.⁶⁰

Specifically the receptor tyrosine kinases AXL and CD271 (together with EGFR and others) have been identified as marker genes for BRAF inhibitor resistance of human melanoma cells in vitro,³⁰ suggesting that targeting AXL^{high} drug resistant melanoma cells putatively affects cells with NCSC-reminiscent features.

Furthermore, targeted as well as immunotherapy have been shown to induce drug resistance, along with acquisition of NCSC-like gene expression programs, that lead to an increased sensitivity to ferroptosis,¹⁰⁶ a type of programmed cell death.¹¹² The study authors subsequently managed to inhibit melanoma dedifferentiation and therapy resistance by addition of ferroptosis-inducing drugs¹⁰⁶ (Figure 2).

The opposite approach of pushing melanoma into a fully differentiated cell fate to circumvent resistance formation, has been achieved in vitro through application of methotrexate (MTX), which activates the expression of microphthalmia-induced TF (MITF), a key regulator of differentiated melanocytes¹¹³. MTX treatment further increased melanoma cell susceptibility to a cytotoxic prodrug (TMECG), activated by tyrosinase, a target of MITF and expressed by fully differentiated melanocytes¹¹¹ (Figure 2).

All in all, these studies have demonstrated that interference with melanoma dedifferentiation or, vice versa, the promotion of a fully differentiated melanoma state can yield increased drug susceptibility and prevention of disease relapse in preclinical models, which make this a highly promising approach for patient therapy.

6 | CONCLUDING REMARKS

Several approaches to target the reemergence of NCSC-like cell states in treatment-resistant melanomas (Figure 2) have shown great promise in preclinical settings. However, further in-depth studies and proper characterization of such NCSC-like melanoma cell states are needed to unravel the exact nature of gene regulatory networks that lead to the most therapy-resilient, and hence aggressive, tumors. Unfortunately, inter-patient and intratumoral heterogeneity has always posed a substantial challenge in melanoma treatment and also complicates the identification of exact NCSC-like programs emerging within melanoma patients.

Currently, most studies performed on NCSC-like cells in melanoma and cited within this review are preclinical studies¹¹⁴ performed on human material in vitro^{30,106} or in murine models, where NC-reminiscent factors were shown to be crucial for tumor formation and disease progression in genetically engineered melanoma models^{24,25,63} as well as in patient-derived xenograft models.^{22,26,28,47} However, extensive analyses of patient materials, which are important to support the clinical relevance of the above discussed findings, are often missing due to limited access to samples reflecting specific stages of melanoma progression. Also, some of the first in-depth single-cell analyses of patient tissues have been single case reports²⁸ rather than studies involving big patient cohorts. Therefore, the possibility remains that the reemergence of aggressive, therapy-resistant NCSC-like cell states observed in

preclinical models does not or not always occur during disease progression in human melanoma patients and that metastatic disease and therapy resistance could still be established by alternative pathways.

Another remaining question concerns whether the mutational landscape predisposes melanoma subtypes toward the potential for dynamic remodeling into NCSC-like cell states. For now, it is unclear whether some of the most frequent melanoma mutations, namely, BRAF^{V600E} and NRAS^{Q61K}, preferably favor the reemergence of NCSC-like cell states. Work by Zon and colleagues has shown that in zebrafish, BRAF^{V600E}-mutated melanomas activate a NCSC progenitor program essential for melanoma initiation,²³ while in NRAS^{Q61K}-mutated melanomas, such a NCSC signature did not emerge at early stages of disease but only after transformation into malignant melanoma.¹¹⁵ Whether this discrepancy is reflective of the human disease physiology remains unclear, since tumor initiation and onset of invasion and metastatic spread cannot be properly monitored or modeled in humans. Furthermore, most preclinical studies that have associated NCSC-like melanoma subpopulations with resistance to therapy have addressed BRAF inhibitor resistance and accordingly used mostly BRAF-mutated melanoma material,^{28,31} leading to a bias toward BRAF- vs NRAS-mutated material under investigation.

In conclusion, future studies including human material from bigger, more representative patient cohorts and collected at different time points of disease progression, specifically also during response to therapies, are needed to address the relevance of NCSC-like melanoma cell states in humans. Hopefully, further single-cell studies will allow us to answer whether NCSC-reminiscent melanoma subpopulations indeed emerge in patients and whether they substantially interfere with melanoma treatment. This could open promising new avenues for designing novel therapies.

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CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

AUTHOR CONTRIBUTIONS

J.D. and L.S. wrote this manuscript.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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